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ABDALLAH HARMACHE,*,[1] PIERRE RUSSO,† FRANÇOIS GUIGUEN,‡ CHRISTIAN VITU,† MICHEL VIGNONI,† MICHÈLE BOUYAC,* CORINNE HIEBLOT,* MICHEL PEPIN,† ROBERT VIGNE,* and MARIE SUZAN*,[2]

*INSERM U372, BP 178, 13276 Marseille Cedex 09, France; †CNEVA Sophia Antipolis, 105 route des Chappes, 06410 Biot, France; and ‡Laboratoire associé INRA-ENV, Ecole Vétérinaire de Lyon, 1 avenue Bourgelat, BP 83, 69280 Marcy l'Etoile, France

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Replication of *vif*- caprine arthritis encephalitis virus (CAEV) is highly attenuated in primary goat synovial membrane cells and blood-derived macrophages compared to the wild-type (wt) virus. We investigated the requirement for CAEV Vif for *in vivo* replication and pathogenicity in goats by intra-articular injection of either infectious proviral DNA or viral supernatants. Wild-type CAEV DNA or virus inoculation induced persistent infection resulting in severe inflammatory arthritic lesions in the joints. We were unable to detect any sign of virus replication in *vif*- CAEV DNA inoculated goats, while *vif*- CAEV virus inoculation resulted in the seroconversion of the goats. However, virus isolation and RT-PCR analyses on blood-derived macrophage cultures remained negative throughout the experiment as well as in joint or lymphoid tissues taken at necropsy. No pathologic lesions could be observed in joint tissue sections examined at necropsy. Goats inoculated with the *vif*- virus demonstrated no protection against a pathogenic virus challenge. These results demonstrate that CAEV Vif is absolutely required for efficient *in vivo* virus replication and pathogenicity and provide additional evidence that live attenuated lentiviruses have to establish a persistent infection to induce efficient protective immunity.

INTRODUCTION

Caprine arthritis encephalitis virus (CAEV) is a member of the lentivirus subfamily of retroviruses which also include human (HIV-1 and HIV-2), simian (SIV), feline (FIV), and bovine (BIV) immunodeficiency viruses, equine infectious anemia virus (EIAV), and ovine visna virus (Narayan and Clements, 1990). These lentiviruses are characterized by the presence of a variable number of genes coding for accessory proteins (*vif, vpr, vpu,* and *nef* in the case of HIV-1), in addition to the genes coding for the structural and the regulatory proteins (Cullen and Greene, 1990). Understanding the role of the different accessory genes of HIV for *in vivo* replication and pathogenesis requires studies of animal models using infectious molecular clones deleted in one or several of these auxiliary genes. Among the animal models for evaluation of HIV infection *in vivo,* infection of common rhesus monkeys with an infectious molecular clone of SIV ($SIV_{mac}239$) results in AIDS-like disease similar to HIV-induced disease in humans (Kestler *et al.,* 1990). Infection of rhesus monkeys with *nef* mutants of $SIV_{mac}239$ revealed the crucial role of Nef in maintaining high virus load and pathogenic potential (Kestler *et al.,* 1991; Daniel *et al.,* 1992). The same kind of studies with *vpr* mutants (Lang *et al.,* 1993; Gibbs *et al.,* 1995; Hoch *et al.,* 1995) and/or with *vpx* mutants of $SIV_{mac}239$ (Gibbs *et al.,* 1995) concluded that these two genes are not required for pathogenicity.

The *vif* gene is the only accessory gene conserved in the genome of the small ruminant lentiviruses, visna virus, and CAEV (Sonigo *et al.,* 1985; Saltarelli *et al.,* 1990; Oberste and Gonda, 1992), suggesting that *vif* is important for natural infection. Recent reports demonstrated that Vif is essential *in vitro* to allow an efficient virus replication in primary lymphocytes infected with HIV-1 and HIV-2 (Gabuzda *et al.,* 1992, 1994; Michaels *et al.,* 1993; Gibbs *et al.,* 1994a; Courcoul *et al.,* 1995), $SIV_{mac}239$ (Gibbs *et al.,* 1994b), and FIV (Shacklett and Luciw, 1994), or primary macrophages infected with CAEV (Harmache *et al.,* 1995a). *In vivo* studies on biological or molecular clones isolated directly or after short-term culture of peripheral blood mononuclear cells (PBMCs) from HIV-1-infected patients revealed that the *vif* open reading frame was conserved with little variability (Sakai *et al.,* 1991; Groenink *et al.,* 1992; Wieland *et al.,* 1994; Sova *et al.,* 1995), suggesting that HIV-1 Vif is required for infection *in vivo.*

The infection of goats with CAEV is a suitable animal model with which to study the ability of deletion mutants to establish persistent infection and pathogenesis. CAEV causes chronic inflammatory disease in persistently infected goats, resulting mainly in mastitis and arthritis (Narayan and Cork, 1985; Cheevers and McGuire, 1988). An infectious molecular clone was derived from the Cork strain of CAEV (Pyper *et al.,* 1986) and its sequence was entirely determined (Saltarelli *et al.,* 1990). Experimental

[1] Present address: University of Washington, School of Medicine, Department of Microbiology, K-451 Health Sciences Center, Box 357740, Seattle, WA 98195-7740.

[2] To whom correspondence and reprint requests should be addressed. Fax: (33) 91 82 60 61: E-mail: msuzan@inserm-U372.univ-mrs.fr.

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infection of goats with this cloned virus reproduced the characteristic features of the natural infection, i.e., persistent infection and induction of inflammatory arthritic lesions in the joints (M. Suzan, unpublished results). We recently described the successful infection of goats after intra-articular inoculation of wild-type CAEV Cork strain proviral DNA, and in the meantime, the accessory role of the CAEV *tat* gene for virus replication *in vivo* was demonstrated (Harmache *et al.,* 1995b). In this study, we investigated the importance of the CAEV *vif* gene *in vivo* by intra-articular inoculation of goats with the *vif* deleted proviral DNA or *vif* deleted virus particles produced on semipermissive primary synovial membrane cells. These experiments established the necessary requirement for a functional CAEV *vif* gene in order to induce infection and pathogenesis.

MATERIALS AND METHODS

CAEV molecular clones

Ligation of two *Hin*dIII fragments, prepared from the 9- and 0.5-kb *Hin*dIII clones, was necessary to generate a full-length wild-type (wt) infectious molecular clone (Pyper *et al.,* 1986; Saltarelli *et al.,* 1990). The *vif*- CAEV infectious molecular clone used in this study was obtained in the same way using the CAEV *vif* Δ2 9-kb *Hin*dIII clone previously described (Harmache *et al.,* 1995a), which harbors a 276-bp deletion in the *vif* gene and has the capacity to encode for the 27 N-terminal amino acids of Vif.

Cells

Primary goat synovial membrane (CFSM1) cells were derived from carpal joints and maintained in culture in Eagle's minimal essential medium (MEM) containing 1% glutamine, penicillin, streptomycin, and 10% fetal calf serum. Peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll-Paque density gradients (Pharmacia) and either processed directly or cultured for 10–12 days in Teflon bags to allow macrophage differentiation and maturation in RPMI 1640 medium supplemented with 1% glutamine, penicillin, streptomycin, 10 m*M* HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 10^{-5} *M* β-mercaptoethanol, and 10% sheep serum. Transfection of CFSM1 cells with the Lipofectin reagent and RT activity measurement in 100-fold concentrated cell-free culture supernatants were previously described (Harmache *et al.,* 1995a).

In vivo experimentation

Ten 9- to 10-month-old goats were obtained from a single CAEV-free breeding (CNEVA Sophia Antipolis) and were inoculated in the left carpus with 1 ml of a mixture of DNA and cationic lipids (DOTAP, Boehringer-Mannheim). Goats 13 and 14 received 100 μg of pRC-CMV DNA (negative control); goats 9 and 18 received 100 μg of wt CAEV proviral DNA; goats 3, 21, 8, and 7 received 100 μg of *vif*- CAEV proviral DNA; goats 153 and 15 were inoculated with 1 ml of wt CAEV viral supernatant containing about 50,000 cpm/ml of RT activity (positive control). Five adult goats were obtained from another CAEV-free breeding (ENV, Lyon) and were injected in the right carpus with 1 ml of medium alone (goat 9315, negative control); 1 ml of wt CAEV viral supernatant (goat 9317, positive control); 1 ml of *vif*- CAEV viral supernatant (goats 9316, 9322, and 9325). Each supernatant was diluted in order to obtain a dose of 20,000 cpm/ml of RT activity. Challenge of this second group of goats was performed on Day 228 postinoculation (p.i.) by injecting, in the opposite carpus (left carpus), 1 ml of wt CAEV viral supernatant containing about 90,000 cpm/ml of RT activity. Goat 9325 was challenged with 1 ml of medium alone. At Day 83 postchallenge (pc), goats were necropsied and samples from different tissues and lymphoid organs were taken and processed for different purposes: synovial membranes were either frozen in liquid nitrogen for histological examination of tissue sections after hematoxylin/eosin staining or put into culture for virus isolation and RT-PCR analyses. RNA was directly extracted from samples of bone marrow and prescapular and retro-mammary lymph nodes for RT-PCR analyses.

Blood samples were taken at regular intervals to determine the serological status of the animals and to isolate PBMCs-derived macrophages for virus isolation and RT-PCR analyses.

PCR and RT-PCR analyses

Total cellular DNA was prepared with the nucleic acid extraction kit Isoquick (Microprobe), according to the manufacturer's protocol. Total cellular RNA was double extracted using the RNAzol (Bioprobe) reagent and protocol. PCR and RT-PCR analyses as well as Southern blot hybridization of the amplified products were performed under the same conditions as previously described (Harmache *et al.,* 1995a). The sequences and positions of the primers used are as follows, according to Saltarelli *et al.* (1990): POL S (sense primer), 5′-GATAGGATAGGAGTGCATTG-3′ (3721 to 3740); POL H (hybridization oligonucleotide), 5′-TATTTCCGAAATATATTTGTC-3′ (3801 to 3781); POL A (antisense primer), 5′-TGAGTCTATGATTCCTCCT-3′ (4020 to 4002); VIF1 (sense primer), 5′-CAGGATCCATGCAAAATTCATCCCGCC-3′ (5006 to 5024), VIF2 (hybridization oligonucleotide), 5′-GGTAATTCTGGTCCAGGT-3′ (5067 to 5050), VIF7 (antisense primer), 5′-CAGGATCCCTTTGAGGCAGTTCTTCAC-3′ (5710 to 5692).

Specific primers were chosen to amplify the caprine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control of the PCRs and RT-PCRs. They are as follows, according to the partial caprine GAPDH sequence (G. Quérat, unpublished results): CGAP1 (sense primer), 5′-GTTCCACTATGATTCCACCC-3′ (25-

44); CGAP2 (hybridization oligonucleotide), 5′-CAGTCAAGGCAAGAGAATGGG-3′ (63-82); CGAPR1 (antisense primer), 5′-TCCCTCCACGATGCCAAA-3′ (406-386).

ELISA and Western blot analyses

ELISA was performed with the Chekit CAEV/MVV test (Behring) against total viral proteins. Results are expressed as the percentage of positive controls established with the formula: (mean tests − mean negative controls)/(mean positive controls − mean negative controls) × 100. According to the manufacturer's instructions, sera with percentages below 30% were considered negative, doubtful when between 30 and 40%, and positive above 40%.

Western blot analysis was performed on viral proteins prepared from sucrose gradient purified virus, separated by electrophoresis on a 12% polyacrylamide–SDS gel, and blotted on nitrocellulose. Sera were tested at a 1/100 dilution and the reaction was revealed with rabbit anti-goat immunoglobulins coupled to peroxidase (Nordic), 1/1000 dilution, and 3,3-diaminobenzidine (Sigma) as substrate.

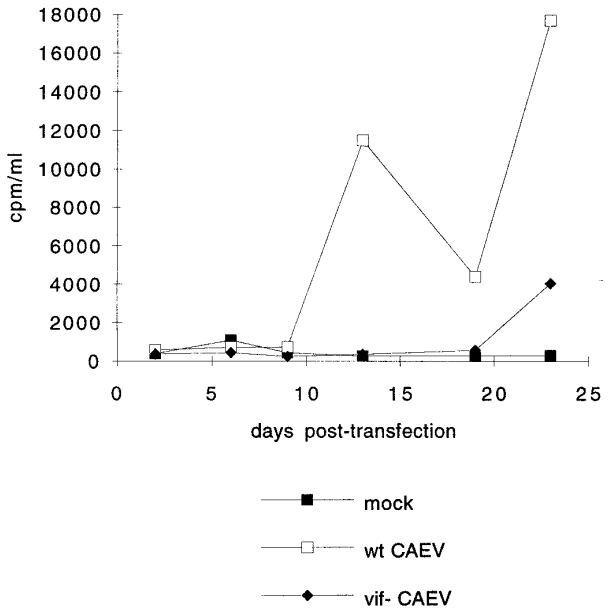

FIG. 1. RT activity measurement in the cell-free supernatants from CFSM1 cells transfected with plasmid DNA (mock), wt or *vif*- CAEV DNA. The medium was changed every 3–4 days and RT activity measured in 1 ml of supernatant 100-fold concentrated.

RESULTS

In vivo inoculation of *vif*- CAEV proviral DNA is unable to induce infection

In order to investigate the importance of the CAEV *vif* gene for virus replication *in vivo,* we chose to inoculate goats with the infectious proviral wild-type DNA (wt CAEV DNA) or DNA deleted in the *vif* gene (*vif*- CAEV DNA). As the generation of an infectious molecular clone of CAEV needs the ligation of two *Hin*dIII fragments, as described under Materials and Methods, part of each DNA preparation (5 μg) used for *in vivo* injection was tested by *in vitro* transfection of primary goat synovial membrane (CFSM1) cells to ensure ligation efficiency. Figure 1 shows the replication profile monitored by RT activity measurement in the cell-free supernatants of the transfected CFSM1 cells. As expected, the *vif*- virus has a very low and slow replication compared to the wild-type virus, corresponding to the attenuated phenotype previously described (Harmache *et al.,* 1995a). The corresponding proviral DNAs were then mixed with an equal volume of cationic lipids and inoculated into goats. Each animal received a single intra-articular injection in the left carpus of 100 μg of pRC-CMV DNA (goats 13 and 14), wt CAEV DNA (goats 9 and 18), or *vif*- CAEV DNA (goats 3, 21, 8, and 7). As positive controls of infection, two goats were inoculated by the same route with wt CAEV viral supernatant (goats 153 and 15). Results of this experiment are summarized in Table 1. Seroconversion determined by ELISA was observed in goats injected with the wt CAEV viral supernatant or proviral DNA at Day 26 p.i. (goats 153 and 15) and Day 49 p.i. (goats 9 and 18), respectively. No antibodies were detected either by ELISA or Western blot assays in the sera or the synovial fluids of the four goats inoculated with the *vif*- CAEV DNA throughout the experiment. Viral sequences detection was performed by PCR analyses through five to seven tests over the 3- to 4-month period of experiment, and was positive, at different time points, only on DNA extracted from PBMCs isolated from wt CAEV viral supernatant or proviral DNA injected animals. These results were confirmed by the analyses performed on the synovial membranes taken at necropsy 3 to 4 months after inoculation.

Part of the membranes were put into culture for virus detection by RT activity measurement into the cell-free culture supernatants and RT-PCR analyses after RNA extraction (Table 1). Negative control goats 13 and 14 which received plasmid DNA produced negative results with all these tests. Virus could be isolated by the two techniques, from the synovial membrane cell cultures of the wt CAEV DNA inoculated goats 9 and 18. Interestingly, synovial membranes from both joints were positive for goat 9, whereas only the left synovial membrane was positive for goat 18, suggesting that in one case, a systemic infection was established allowing virus detection in the joint opposite to the inoculation site. Virus isolation was negative for the three *vif*- CAEV DNA inoculated goats tested (3, 8, and 7). RT-PCR analysis using primers allowing detection of the spliced *rev* mRNA showed a positive signal only for RNA extracted from synovial membrane cells of goats 9 and 18 (wt CAEV DNA). Another part of the different synovial membranes was frozen and tissue sections were examined for the presence of inflammatory lesions after hematoxylin/eosin staining.

TABLE 1

Experimental Infection of Goats by Intra-articular Inoculation of wt or *vif*- CAEV Proviral DNA or Viral Supernatants

	Inoculation[a]									
	Plasmid DNA		wt CAEV viral supernatant		wt CAEV proviral DNA		*vif*- CAEV proviral DNA			
Animal No.	13	14	153	15	9	18	3	21	8	7
ELISA[b]										
Serum	−	−	+	+	+	+	−	−	−	−
Synovial fluid	−	−	+	+	+	+	−	−	−	−
Western blot[c]										
Serum	−	−	+	+	+	+	−	−	−	−
Synovial fluid	−	−	+	+	+	+	−	−	−	−
Virus detection in PBMCs										
PCR[d]	−	−	+	+	+	+	−	−	−	−
Virus detection in synovial membrane cells										
RT activity[e]	−	NT	NT	NT	+	+	−	NT	−	−
RT-PCR	NT	−	NT	NT	+	+	−	NT	−	−

[a] Each goat received a single intra-articular inoculation in the left carpus of either 100 μg DNA complexed to cationic lipids or 1 ml of wt viral supernatant (50,000 RT cpm).

[b] Seroconversion was determined by ELISA using a commercial kit (Behring). Values 40% above positive control were scored as positive (+) and otherwise considered negative (−).

[c] Western blot assays were performed on total viral proteins separated on a 12% polyacrylamide–SDS gel. Sera or synovial fluids were tested at a 1:100 dilution. (+) denotes the presence of antibodies against Gag and Env proteins; (−) indicates that no specific bands were observed.

[d] DNA was extracted from 10^7 freshly isolated PBMCs and submitted to PCR amplification using either *pol* (POLS/POLA)- or *vif*-specific primers (VIF1/VIF7).

[e] Part of the synovial membranes taken at necropsy were put into culture. Virus detection was performed by direct isolation from culture supernatants (RT activity) or by RT-PCR analyses after RNA extraction, using primers specific for the spliced *rev* mRNA. NT, not tested.

Characteristic pictures of tissue sections from goats 13 (negative control), 9 (wt CAEV DNA), and 3 (*vif*- CAEV DNA) are shown in Fig. 2. While the synovial membranes of goats 13 and 3 presented a normal aspect (Figs. 2a and 2c, respectively), severe inflammatory lesions could be observed in tissue sections from goat 9 (Fig. 2b), with extensive mononuclear cell infiltration comprising plasmocytes.

vif- virus is able to induce seroconversion but not persistent infection of goats

In this experiment, goats received a single intra-articular injection in the right carpus of 1 ml of viral supernatants, each containing 20,000 cpm/ml of RT activity, corresponding to 3.3×10^4 TCID50 for the wt virus and 5×10^3 TCID50 for the *vif*- virus. Although not identical, the number of infectious particles injected into goats was above the goat infectious dose (M. Suzan *et al.,* unpublished results). One negative control goat, 9315, was injected with 1 ml of culture medium, the positive control goat, 9317, was inoculated with wt virus supernatant, and three goats, 9316, 9322, and 9325, received *vif*- virus supernatant. The follow-up of the antibody response determined by ELISA against total viral proteins is shown in Fig. 3. Sera with values above 40% of the positive control were considered positive. All the animals, except the negative control 9315, seroconverted at Day 21 (9317, positive control) or Day 28 p.i. (9316, 9322, and 9325). The *vif*- virus inoculated goat 9325 exhibited the highest level of antibody response, equivalent to the positive control goat 9317, and, although the level decreased, it remained positive until Day 221 p.i. Sera titers from the two other *vif*- virus injected goats, 9316 and 9322, dropped below the limit of positivity from Day 102 p.i. In order to compare the reactivity profile of these antibodies against the different viral proteins, sera at Day 7 and Day 221 p.i. were analyzed in Western blot assays (Fig. 4). All sera taken at Day 7 p.i. showed no specific bands (lanes 1, 4, 7, 10, and 13) as did serum from goat 9315 at Day 221 p.i. (lane 2) and negative control serum (lane −C). Sera from inoculated goats revealed different patterns of reactivity at Day 221 p.i.: The positive control goat 9317 (lane 5) developed antibodies against all the major forms of Gag proteins, either precursors (Pr55gag, Pr48gag, Pr38gag) or mature forms (p28, p18, and p14.5), as in positive control serum (lane +C). Anti-p28 antibodies could be observed in sera from *vif*- virus injected goats whereas antibodies against the precursor forms of Gag could not be detected (lanes 8, 11, and 14). Anti-p18 antibodies were weakly detected in sera from goat 9316 (lane 8). Anti-p14.5 antibodies were present in sera from goat 9316 (lane 8) and 9325 (lane 14) but not in serum from goat 9322 (lane 11). Under our test conditions, it was not possible to detect the envelope proteins.

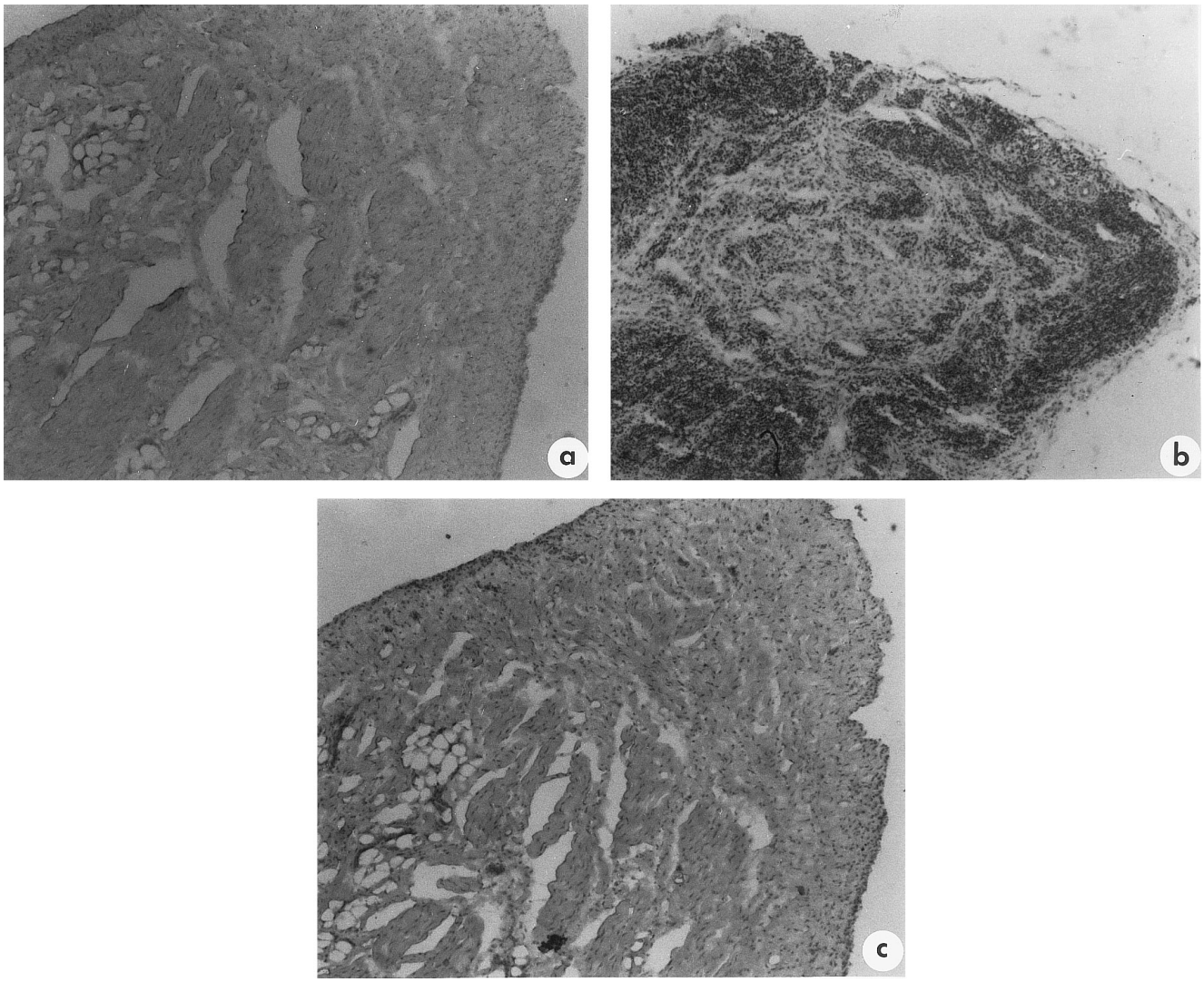

FIG. 2. Histopathology of the synovial membranes of negative control goat (a) or after wt (b) or *vif*- (c) proviral DNA intra-articular inoculation. Synovial membranes samples were taken at necropsy and frozen sections were stained with hematoxilin/eosin.

Virus isolation was possible by monitoring RT activity in the culture supernatants of blood-derived macrophages taken at Weeks 4 and 11 p.i. from the positive control goat 9317 and became negative thereafter. Virus isolation was negative for each *vif*- virus inoculated goat in all the assays. Since only a small percentage of blood monocytes/macrophages are infected by CAEV (Narayan *et al.,* 1983; Gendelman *et al.,* 1986) and since direct virus isolation from cultured macrophages is not a sufficiently sensitive technique, we performed virus detection by RT-PCR. Results are summarized in Table 2. Over the 31-week period before challenge, seven RT-PCR assays were performed on RNA extracted from blood-derived macrophages. A positive signal could transiently be observed in samples from the wt immunized 9317 goat at Weeks 2, 4, and 11, whereas virus detection in the *vif*-CAEV inoculated goats, 9316, 9322, and 9325, remained negative. As a control, amplification of the housekeeping gene GAPDH provided the same intensity signal in each sample (data not shown).

vif- CAEV inoculated goats are not protected against a pathogenic challenge

Challenge was performed at Week 32 p.i. by intra-articular injection in the left carpus of 1 ml of wt virus supernatant, equivalent to 90,000 cpm/ml of RT activity. The *vif*- virus injected goat 9325 received 1 ml of medium alone (inoculated control). Goat C70 was a naive animal that was included in the experimental group the day of challenge as a positive control of infection and thus received only the challenge dose. The serological status of the animals was determined by ELISA at Day 83 post-challenge (pc, Fig. 3). Goat 9317 as well as goats 9316 and 9322 (inoculated challenged) exhibited ELISA values higher than those determined at Day 221 p.i., indicating efficient stimulation of the immune system, as was also the case for goats 9315 (mock inoculated challenged) and C70 (uninoculated challenged). The inoculated control goat 9325 was still positive but with a decreased ELISA value. The Western blot profile of these sera was

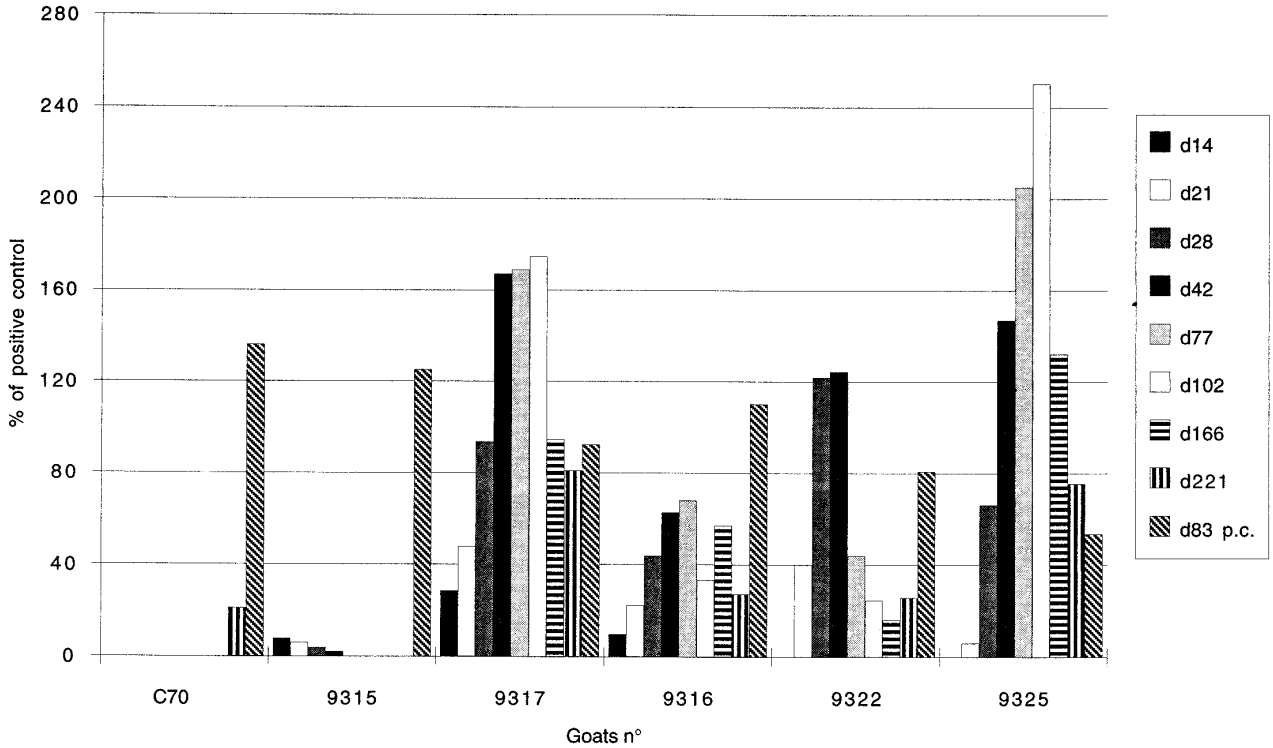

FIG. 3. Follow-up of the antibody response after wt or *vif*- virus inoculation and pathogenic challenge. Goats received at Day 0 one intra-articular injection of 1 ml of medium (9315), wt (9317), or *vif*- (9316, 9322, 9325) viral supernatants. Challenge was performed at Day 228 with wt virus, except for goat 9325. Goat C70 was inoculated at Day 228, as a positive control of challenge. Sera were taken at the indicated time points postinoculation and at Day 83 postchallenge (pc). They were considered positive when the ELISA value was 40% above that of the positive control.

determined (Fig. 4). All the sera presented a similar and complete pattern of reactivity against the Gag proteins (lanes 3, 6, 9, and 12), as described above. The *vif*- virus infected mock challenged goat 9325 only showed antibodies against p28 and p14.5 (lane 15).

Challenge virus detection was positive at different times pc for the five wt challenged goats (data not shown for goat C70), either by direct isolation from blood-derived macrophages or by RT-PCR on macrophage RNA, while the mock challenged goat 9325 was negative (Table 2). This detection was transient since all the samples were negative at week 12 pc, when the animals were necrop-

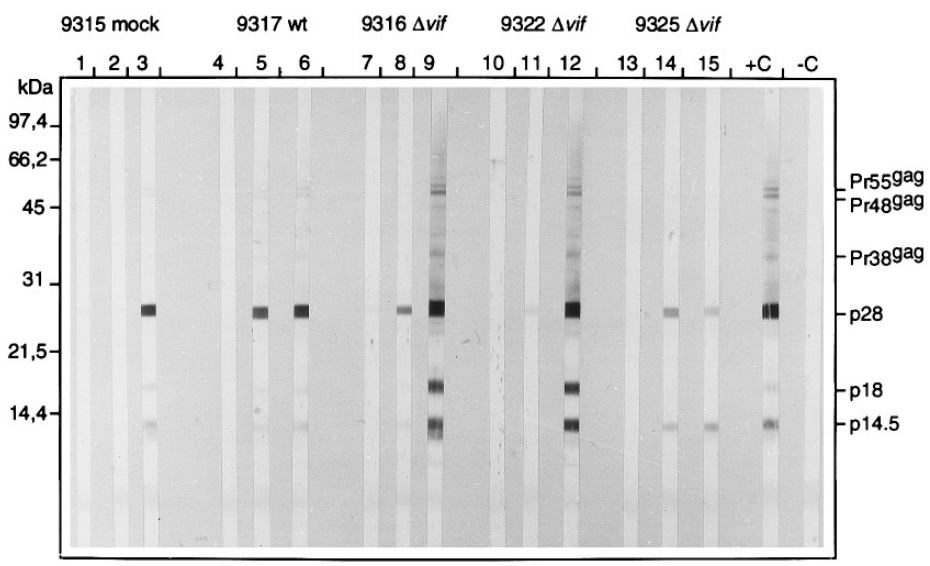

FIG. 4. Western blot analysis of the antibody response pattern after wt or *vif*- virus injection and after virulent challenge. Sera were taken at Day 7 (lanes 1, 4, 7, 10, and 13), Day 221 p.i. (lanes 2, 5, 8, 11, and 14), or Day 83 postchallenge (3, 6, 9, 12, and 15). All the animals were challenged, except goat 9325. +C, positive control serum; −C, negative control serum. Molecular weight markers are indicated on the left.

TABLE 2

Virus Detection by RT-PCR from Blood-Derived Macrophages of Goats Inoculated with wt or *vif*- CAEV before and after Challenge with Pathogenic CAEV

Group	Animal No.	p. i p.c.	1	2	4	6	11	14	31	32 0	34 2	36 4	38 6	39 7	40 8	44 12
Mock inoculated/wt challenged	9315		−	−	−	−	−	−	−	−	NT	+	+	+	−	−
Inoculated control vif-/mock	9325		−	−	−	−	−	−	−	−	−	−	−	−	−	−
Inoculated challenged wt/wt	9317		−	+	+	NT	+	−	−	−	+	NT	+	+	+	−
vif-/wt	9316		−	−	−	−	−	−	−	−	+	NT	+	−	+	−
vif-/wt	9322		−	−	−	−	−	−	−	−	+	+	+	+	+	−

RT-PCR analysis on blood derived macrophages taken at different weeks postinoculation (p.i.) or postchallenge (pc, boldface entries). RNA was extracted from blood derived macrophages after 10 days of culture in Teflon bags. RT products were submitted to PCR amplification with the *pol*- or *vif*- specific primers (POLS/POLA or VIF1/VIF7). Southern blots of the amplified products were hybridized with the ^{32}-P-labeled POLH or VIF2 oligonucleotide probe. NT, not tested.

sied. We already observed this transient virus detection after CAEV wt inoculation of goat 9317 and in several other *in vivo* experimental CAEV infections (Harmache *et al.,* unpublished results). Different tissue samples were taken at necropsy and analyzed by RT-PCR after direct RNA extraction. Southern blot of the products amplified with the *pol*- specific primers (POLS/POLA) was hybridized with a ^{32}P-labeled internal oligonucleotide probe (POLH). Results are shown in Fig. 5. Except for the right synovial membrane sample of goat 9316 (lane c), challenge virus could be detected in any of the tissue tested, i.e., left (lanes a) or right (lanes c) synovial membranes, bone marrow (lanes b), left (lanes d) or right (lanes e) retro-mammary lymph nodes, and left (lanes f) or right (lanes j) prescapular lymph nodes. Once again, samples from the *vif*- virus infected, mock challenged goat 9325 produced only negative signals, whereas the internal control GAPDH was positive.

Tissue sections from the frozen part of the synovial membranes were examined for the presence of histological lesions after hematoxylin/eosin staining (Fig. 6). As already observed in the first experiment of this study (Figs. 2a and 2c), synovial membranes from mock or *vif*- virus infected goat 9325 were normal (Figs. 6a and 6c). Severe inflammatory lesions with massive infiltration of macrophages and lymphocytes were observed in joints of the mock infected, wt virus challenged goat 9315 (Fig. 6b) and goat 9317 (not shown), whereas joints from *vif*- virus infected, wt virus challenged goats developed lesions but to a somewhat lesser extent, as shown by a representative picture in Fig. 6d.

DISCUSSION

The CAEV *vif*- virus has been proven able to replicate *in vitro* in primary synovial membrane cell cultures and

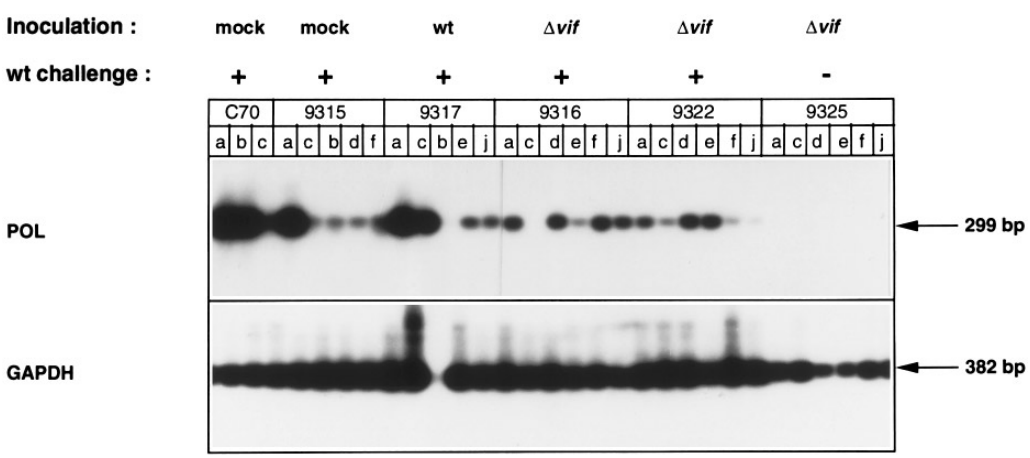

FIG. 5. Challenge virus detection by RT-PCR analysis on different tissues taken at necropsy. RNA was directly extracted from tissue samples and RT products were submitted to PCR amplification using the *pol*-specific primers (POLS/POLA). Southern blot of the amplified products was hybridized with the ^{32}P-labeled POLH oligonucleotide probe. Amplification of GAPDH mRNA was used as an internal control. Tissues analyzed were synovial membranes from the left (a) or right (c) joint; bone marrow (b); left (d) or right (e) retro-mammary lymph node; and left (f) or right (j) prescapular lymph node.

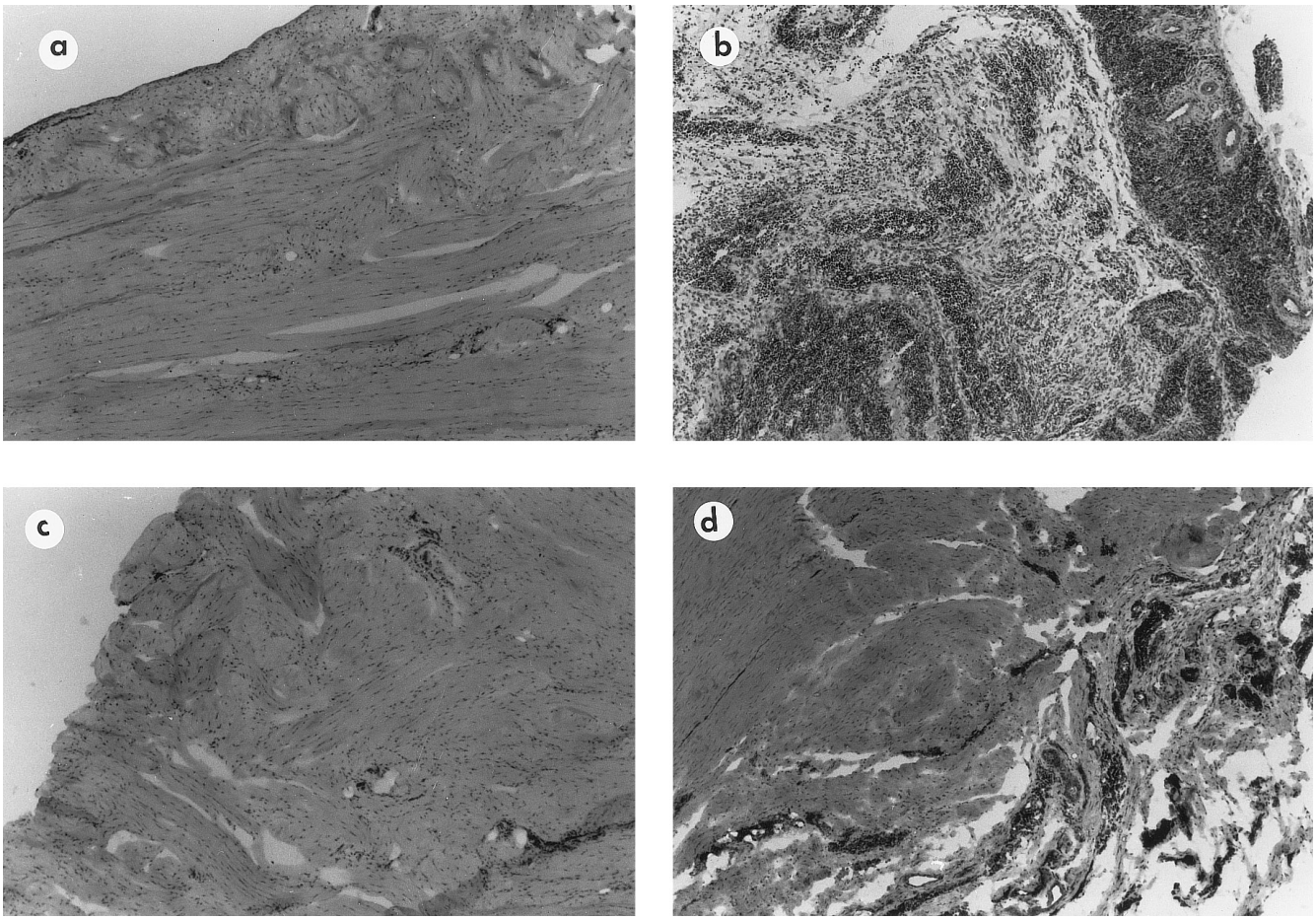

FIG. 6. Histology of synovial membranes from a mock infected goat (a), mock infected, wt challenged goat (b), *vif*- virus inoculated goat (c), and *vif*- virus injected, wt challenged goat (d). Sections were prepared as in Fig. 2.

primary blood derived macrophages, with an attenuated phenotype compared to the wt virus (Harmache *et al.,* 1995a). These cells represent the natural target cells for CAEV infection in vivo (Klevjer-Anderson and Anderson, 1982; Narayan *et al.,* 1983). This paper reports the necessary requirement for a functional *vif* gene of the lentivirus CAEV for *in vivo* induction of persistent infection and pathogenesis. Infectious molecular clones of lentiviruses are important tools for determining the role of the so-called "nonessential" genes in the molecular mechanisms of pathogenesis, but also for therapy development and vaccine research (Desrosiers, 1992; Gibbs *et al.,* 1994a, 1994b). Demonstration that an infectious molecular clone of SIVmac could induce an AIDS-like disease in rhesus monkeys (Kestler *et al.,* 1990) as well as subsequent studies *in vivo* using nonessential gene-deletion mutants of this clone (Lang *et al.,* 1993; Gibbs *et al.,* 1995; Hoch *et al.,* 1995) provided new insights into the relative importance of such genes in virus replication, persistence, and pathogenesis.

Some reports described the successful infection of animals upon injection of proviral DNA from different retroviruses such as SIVmac (Letvin *et al.,* 1991) or bovine leukemia virus (Willems *et al.,* 1992), and the *in vivo* study of viral infectivity of genetic mutants of BLV has also been reported (Willems *et al.,* 1993). We previously demonstrated that inoculation into goats of proviral DNA from the wt or *tat* deleted infectious molecular clone of CAEV resulted in persistent infection and disease progression (Harmache *et al.,* 1995b). The same approach was thus used to study the impact of the *vif* gene deletion on virus replication *in vivo.* No evidence of virus replication could be obtained by different techniques (ELISA, virus isolation, PCR, and RT-PCR), in goats inoculated with the *vif*- proviral DNA whereas inoculation of the wt proviral DNA established infection leading to the induction of inflammatory lesions in the joints. These results demonstrated that *in vivo* replication of CAEV was impaired as a result of the *vif* gene deletion; thus, experimental CAEV infection is a relevant animal model for the understanding of the contribution of different genes to infection and pathogenesis. Because DNA inoculation in the presence of cationic lipids could be considered an *in vivo* transfection, without any cell targeting specificity, we performed a second *in vivo* experiment in which goats received intra-articular injection of CAEV *vif*- viral supernatants in order to increase the percentage of infected specific target cells. Virus isolation as well as RT-PCR analyses

produced only negative results during the time course of the experiment, whereas CAEV wt inoculated goats were transiently positive by these assays. These transient virus isolations are not the sign of a cleared infection since the animals developed inflammatory lesions in the joints and RT-PCR analyses of different lymphoid tissues at necropsy allowed virus detection (see also Harmache *et al.,* 1996). Most probably they reflect a low viral load in the monocytic compartment at the blood level. The only evidence of virus expression in *vif*- virus inoculated goats was provided by the seroconversion as detected by ELISA, indicating that enough viral proteins were expressed to stimulate the antibody response. We observed that sera from *vif*- virus inoculated goats contained antibodies directed mainly against two of the mature forms of the Gag protein, p28 and p14.5, whereas the p18 and the precursor forms were additionally detected by sera from wt CAEV infected goats. Goat 9325 (inoculated control) exhibited the highest level of antibody response. However, the incomplete Western blot profile produced by its antibodies remained unchanged throughout the experiment. This result was rather unexpected since it was previously reported that inoculation of purified formalin-inactivated wt CAEV or SIV virions resulted in a strong antibody response exhibiting a complete reactivity profile in Western blot assay (Murphey-Corb *et al.,* 1989; Vitu *et al.,* 1993). Several hypotheses could be proposed. The antibody response in *vif*- virus injected goats could have been raised against the viral proteins in the inoculum and in this case would reflect an abnormal composition of *vif*- CAEV virions. Recent papers characterized an abnormal composition of HIV-1 *vif*- virions which contained higher levels of Pr55gag than wt virus particles (Borman *et al.,* 1995; Simm *et al.,* 1995) but also an increased quantity in p17 and p24 and a reduced quantity of envelope proteins (Borman *et al.,* 1995), as already proposed by other authors (Sakai *et al.,* 1993). Alternatively, a weak expression of viral proteins due to a very low level of *vif*- virus replication could generate this kind of antibody reactivity profile, as previously suggested in the case of the attenuated live $SIV_{mac\text{-}1A11}$ (Marthas *et al.,* 1990).

Since *vif*- virus stocks produced on semipermissive cells retained some infectious capacity, we think that infection of natural target cells could have occurred upon *vif*- virus inoculation but most likely produced quantitatively fewer viral particles than the wt virus, as described for an *in vitro* case (Harmache *et al.,* 1995a). Virions produced after this first round of infection were either unable to reinfect other target cells, thus resulting in an abortive infection, or maintained an undetectable viral load. RT-PCR analyses on different tissues or lymph nodes draining the inoculation sites (prescapular) or target organ (retro-mammary) taken at necropsy revealed only wt challenge virus. However, we cannot exclude the possibility that any *vif*- virus replication took place in these organs at earlier time points after inoculation. The *vif*- virus inoculated goats were not protected against the virulent challenge, indicating that the induced antibody response is not involved in protective immunity. By comparison, *in vivo* experiments using proviral DNA or virus inoculation of *tat*- CAEV revealed that this attenuated deletion mutant was able to induce some protective effect against pathogenic challenge (Harmache *et al.,* 1996, and manuscript in preparation). These results could be put together with those reported in the SIVmac model in which the resistance to challenge infection was inversely related to the degree of attenuation of the immunizing clone (Lohman *et al.,* 1994; Denesvre *et al.,* 1995). Deletion of the *vif* gene in the CAEV genome generates very attenuated virus particles, unable to persistently replicate *in vivo* and thereby unable to induce an efficient stimulation of the host immune response conferring protective immunity.

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